INCIDENCE OF MUPIROCIN RESISTANCE IN *STAPHYLOCOCCUS PSEUDINTERMEDIUS* ISOLATED FROM A HEALTHY DOG

A Thesis

by

STACEY MARIE GODBEER

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Chair of Committee,	Sara D. Lawhon
Committee Members,	Michael F. Criscitiello
	Wesley Bissett
Head of Department,	Linda L. Logan

August 2013

Major Subject: Biomedical Sciences

ABSTRACT

Mupirocin is a bacteriostatic antibiotic that is used to decolonize people who carry methicillin-resistant staphylococci, primarily methicillin-resistant *Staphylococcus aureus* (MRSA). Mupirocin reversibly binds to bacterial isoleucyl tRNA synthetase to disrupt protein synthesis. Resistance to mupirocin is due either to a point mutation to the *ileS* gene that encodes the isoleucyl tRNA synthetase, classified as low-level mupirocin resistance; or, bacteria may obtain a plasmid that carries the *ileS2* gene encoding an alternate isoleucyl tRNA synthetase, conferring high-level resistance. Mupirocin resistance plasmids contain insertion sequence (IS) 257 repeats, into which the *ileS2* gene is inserted. Such plasmids have been characterized by their IS257-*ileS2* junctions in both *S. aureus* and, recently, in *Staphylococcus pseudintermedius* in a dog from Croatia. The primary goals of this study were to determine the prevalence of mupirocin resistance in isolates of *S. pseudintermedius* in Texas, to determine whether resistance was due to point mutations in the native *ileS* or due to carriage of mupirocin resistance plasmids, and to characterize the structure of the mupirocin resistance genes carried on plasmids.

In this study, 572 *S. pseudintermedius* isolates, collected from veterinary patients from across Texas were screened for their susceptibility to low levels of mupirocin. Of these isolates, only one out of 572 (0.17%) tested positive for mupirocin resistance and was found by polymerase chain reaction (PCR), using previously published primers mupA and mupB, to have a 458 bp fragment and, with primers M1 and M2 to have a

237 bp fragment, indicating the presence of the high-level mupirocin resistance gene, *ileS2*. The arrangement of the IS257-*ileS2* junctions was then analyzed by PCR and the products, bands at 1816 bp for primers *ileS2*-5' and $IS257_R$ and at 1127 bp for primers *ileS2*-3' and $IS257_F$, which are consistent with the amplification pattern for an S2 plasmid, were cloned into a plasmid, pT7Blue, and sequenced for comparison to published sequences in GenBank. BLAST analyses in NCBI, comparing the isolate to recently published sequences for mupirocin-resistant *S. pseudintermedius* isolated from a dog with pyoderma in Croatia, indicate a 100% similarity to the upstream junction, JX186508, and 97% to the downstream junction, JX186509.

DEDICATION

I dedicate this work to my family: to my parents, Dennis and Linda, for their encouragement and unwavering support throughout the years, to my husband, Travis, for his love and patience and for always believing in me, and to my daughter, Riley, for inspiring me to achieve my dreams so I can support her in reaching hers. I love you all and I never could have done this without you.

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Lawhon, and my committee members, Dr. Criscitiello, and Dr. Bissett and collaborator Dr. Randi Gold, for their guidance and support throughout the course of this research. For their expert technical assistance in the laboratory, I also thank Elena Gart, Jacob Villegas, and Courtney Brake.

Thanks also go to my friends and colleagues and the department of Veterinary Pathobiology faculty and staff for making my time at Texas A&M University a great experience. I also want to extend my gratitude to Dr. Randi Gold, specifically for supplying funds from her Texas A&M University College of Veterinary Medicine and Biomedical Sciences Postdoctoral Research Grant to help support this study.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

NOMENCLATURE

C	Celsius
CoNS	Coagulase-negative staphylococci
IS	Insertion sequence
MIC	Minimum inhibitory concentration
MLST	Multi-locus sequence typing
MRSA	Methicillin-resistant *Staphylococcus aureus*
MRSP	Methicillin-resistant *Staphylococcus pseudintermedius*
MSSA	Methicillin-susceptible *Staphylococcus aureus*
MSSP	Methicillin-susceptible *Staphylococcus pseudintermedius*
PCR	Polymerase chain reaction
PFGE	Pulse field gel electrophoresis
RFLP	Restriction fragment length polymorphism
SCC*mec*	Staphylococcal cassette chromosome *mec*
SIG	*Staphylococcus intermedius* group

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Staphylococci

Staphylococci include a clinically important group of potentially pathogenic Gram-positive bacteria affecting both humans and animals. The most well known species is *Staphylococcus aureus,* which is renowned as an opportunistic pathogen capable of causing illnesses ranging from minor skin infections to life-threatening conditions, like toxic shock syndrome and endocarditis (12). *S. aureus* has reached the attention of the public eye because it is the most common cause of hospital acquired infection and often carries resistance to multiple antibiotics, making it difficult to treat. However, other staphylococci share some of the same attributes that make *S. aureus* successful as a pathogen. For example, *Staphlylococcus pseudintermedius* is capable of producing some of the virulence factors such as coagulase, thermonuclease, proteases, leukocidin, hemolysins, exfoliative toxins, enterotoxins, and pyrogenic toxin superantigens (14, 18). Likewise, *S. pseudintermedius* can also carry some of the same antibiotic resistance genes and can produce biofilms (14). In contrast, staphylococci differ in the surface proteins that interact with the host, causing species variation from host to host, with some species showing higher degrees of host specificity than others. To illustrate, *S. aureus* is the predominant staphylococcal species in humans, horses, and ruminants, *S. pseudintermedius* is the prevalent species in dogs, and *Staphylococcus intermedius* predominates in pigeons (40). However, *S. aureus* is highly adaptable,

colonizing a variety of mammals, reptiles, and birds, and has been documented in dogs, cats, pigs, ruminants, horses, rabbits, and poultry (14). Overall, *Staphylococcus* is a genus that includes successful opportunistic pathogens affecting humans and animals.

One clinically relevant group of staphylococci is the *Staphylococcus intermedius* group (SIG), which is known to cause infections in animals and occasionally in humans. Historically, *S. intermedius* was identified as the causative agent of pyoderma in dogs and had been isolated from dogs, cats, pigeons, mink, and horses (39). Recent investigations into the genetics of *S. intermedius* revealed that rather than a single species, there was a cluster of closely related species which became known as the SIG (48). This group was delineated after the recognition of *S. pseudintermedius* in 2005 (10) and subsequent investigations into molecular typing methods (48). Isolates that had previously been phenotyped as *S. intermedius* were then reclassified into the SIG, which is comprised of *S. intermedius*, *S. pseudintermedius*, and *Staphylococcus delphini* (8, 48). Sasaki et al. demonstrated that *S. intermedius* could be distinguished from *S. pseudintermedius* and *S. delphini* biochemically (39). Later, Bannoehr and colleagues were able to differentiate *S. pseudintermedius* from *S. delphini* using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (4). Due to the discovery of *S. pseudintermedius* and advancements in genetic testing it was discovered that *S. pseudintermedius*, and not *S. intermedius*, was the major pathogenic species in dogs and cats (48). Thus, it has been proposed that isolates previously identified as *S. intermedius* from dogs, be considered *S. pseudintermedius* unless genetically proven to be another member of the SIG (48). There is still debate over the primary staphylococci in cats though, as evidence is present for both *S. aureus* and *S. pseudintermedius* (6). Identification of bacteria in clinical

microbiology laboratories was originally based on Gram-stain reaction and reaction to biochemical tests. One of the most important tests used for identification of staphylococci was their ability to initiate plasma clots through activity of the enzyme coagulase. Both *S. pseudintermedius* and *S. aureus* produce coagulase. Because differentiation between members of the SIG by phenotypic tests is limited, accurate commercial tests for identification of *S. pseudintermedius* are not available, and *S. pseudintermedius* is a relatively new species, it is commonly misidentified as *S. intermedius* or *S. aureus*; therefore, occurrence of *S. pseudintermedius* is likely underestimated in human infections (48, 50). However, if the protocol of a veterinary laboratory is to presume that coagulase-positive staphylococcal isolates cultured from dog and cat infections are *S. pseudintermedius*, then it is equally likely that the incidence of *S. pseudintermedius* will be overestimated due to misidentification of *S. aureus* or *S. intermedius* as such. Therefore, in assessing the occurrence of *S. pseudintermedius*, laboratory capabilities and protocols must be taken into consideration.

It remains common in veterinary labs to identify staphylococci based on their ability to produce coagulase. *S. aureus*, *S. intermedius*, *S. delphini*, *S. pseudintermedius*, and *Staphylococcus schleiferi* subspecies *coagulans* are all coagulase-positive (39). Staphylococci that do not produce this enzyme are termed coagulase-negative staphylococci (CoNS). Members of the CoNS include *Staphylococcus epidermidis*, the most common *Staphylococcus* found on human skin, *Staphylococcus haemolyticus*, and *Staphylococcus saprophyticus*, to name a few (37). Until recently, the CoNS were thought to be innocuous. However, *S. epidermidis* is now believed to cause most cases of device-related infection in hospitals, *S. haemolyticus* is known to cause septicemia,

endocarditis, and other infections, and *S. saprophyticus* has been found to be the second most common cause of urinary tract infections in women of child-bearing age (37). *S. epidermidis* is the best studied of the CoNS. The characteristics that make *S. epidermidis* of clinical concern are its ability to form biofilms in medical devices and its ability to harbor antimicrobial resistance determinants (37). The more research is done on staphylococci, the more investigators realize what a formidable group of pathogens they are.

Staphylococcus pseudintermedius

S. pseudintermedius in dogs is somewhat analogous to *S. aureus* in humans. Both organisms are commensal, living on the skin and/or mucous membranes of their respective hosts, and both are capable of causing infection when the epithelium is disrupted or during times of weakened immune response. Of the staphylococci, *S. aureus* is considered the most significant human pathogen; notably it is a common cause of minor skin infections and can penetrate the epithelium at surgical incisions, intravenous catheter sites, and injection sites (12). Likewise, *S. pseudintermedius* is the major pathogenic species isolated in canine pyoderma, ear infections, and is also capable of causing post-surgical infections (14, 16, 48). Both organisms are also capable of transmission from humans to animals and vice versa. *S. aureus* has a wide range of host species, but *S. pseudintermedius* is typically associated with dogs. However, *S. pseudintermedius* does have zoonotic potential, reportedly causing human infections in dog bite wounds (43), sinusitis (23, 42), bacteremia (7), and pneumonia (17). Humans

can also be nasal carriers of *S. pseudintermedius*, although it is not as common. In a study of dogs with deep pyoderma, it was found that dog owners frequently carried the same strain of *S. pseudintermedius* as their dog, but that this colonization was only transient as owners were no longer culture-positive after the dog's infection resolved (19). Veterinary dermatologists and small animal clinical staff are also nasal carriers of *S. pseudintermedius* (31).

Both *S. aureus* and *S. pseudintermedius* are capable of causing nosocomial and community-acquired infections in their respective normal host species. According to Lindsay, *S. aureus* is the most common cause of hospital acquired infection and the incidence of community acquired *S. aureus* is increasing (27). One of the greatest complications of *S. aureus* and *S. pseudintermedius* infection is drug resistance, specifically methicillin-resistance. Although these methicillin-resistant staphylococci are no more virulent than non-resistant strains, they are more difficult to treat clinically and can therefore spread more readily in hospitals or in community settings where people are in close contact with one another (27). Zubeir et al. demonstrated by pulse field gel electrophoresis (PFGE) using *ApaI* and *SmaI* restriction enzymes that ten isolates of methicillin-resistant *S. pseudintermedius* (MRSP) taken from animals at a veterinary clinic were indistinguishable, suggesting that a single bacterial clone was distributed among these animals either from the clinic or in the pet population (54). Additionally, both *S. aureus* and *S. pseudintermedius* are capable of acquiring multiple antimicrobial resistance determinants, making them difficult to treat. This, compounded with the fact that staphylococci are capable of transferring resistance determinants between species,

means that *S. pseudintermedius* in animals could serve as a reservoir for antimicrobial resistance for *S. aureus* in humans and vice versa.

History of Drug Resistance in Staphylococci

Shortly after the introduction of the antibiotic penicillin in the 1940s, penicillin-resistant staphylococci were recognized (29). It was discovered that these *S. aureus* strains possessed the *blaZ* gene encoding the enzyme β-lactamase, which hydrolyzes the β-lactam ring of penicillin, rendering it inactive (29). In 1961, the first semisynthetic penicillinase-resistant penicillin, methicillin, was introduced; shortly thereafter, methicillin-resistant staphylococci emerged as well (29). Resistance to β-lactam antibiotics, including penicillin, methicillin, and cephalosporins, in staphylococci is conferred by the *mecA* gene which encodes the penicillin-binding protein, PBP2a (1). Penicillin-binding proteins catalyze transpeptidation, necessary for cross-linkage of peptidoglycan chains during cell wall synthesis in Gram-positive bacteria; importantly, PBP2a has a low affinity for β-lactam antibiotics, enabling cell wall synthesis to continue even in high concentrations of such antimicrobials (29). Staphylococci carrying the *mecA* gene are termed methicillin-resistant. β-lactam antimicrobials are often used empirically and are considered first-line of defense treatment for staphylococcal infections in people and pyoderma in dogs because they have a low risk of adverse effects and good tissue penetration. In general, this practice is effective and these drugs generally eliminate methicillin-susceptible staphylococci; unfortunately though, extensive use of β-lactam antibiotics can select for methicillin-resistant strains (6). In

recent years, both methicillin-resistant *S. aureus* (MRSA) infections and methicillin-resistant *S. pseudintermedius* (MRSP) infections have increased (14). The incidences of both hospital-associated MRSA and community-acquired MRSA are increasing; additionally, statistics show that 20–25 percent of healthy humans carry *S. aureus* all the time and 55–60 percent carry *S. aureus* intermittently (12). In healthy dogs, MRSP carriage can be as high as 6.2% (16/258) (31). Historically, MRSP isolates remained susceptible to one or more antimicrobials outside of the β-lactam antibiotics. Among the drugs alternatively selected to treat MRSP infections are fluoroquinolones, macrolides, lincosamides, chloramphenicol, aminoglycosides, and rifampin. Unfortunately, resistance to each of these classes of antimicrobial drugs in staphylococci has increased in recent years and there are few choices for systemic therapy for MRSP infections (36).

Staphylococcal Cassette Chromosome *mec* (SCC*mec*)

Characterization of MRSP is done by sequence type determination and by typing the mobile element carrying the *mecA* gene that confers β-lactam antimicrobial resistance. The *mecA* gene is carried on the mobile genetic element, staphylococcal cassette chromosome *mec* (SCC*mec*). SCC elements carry *ccr* genes which encode the enzymes necessary for excision and integration of the element into the *orfX* gene of the staphylococcal chromosome (26). SCC*mec*, has been categorized into eight different types, SCC*mec* types I – VIII, in *S. aureus* (47). In *S. pseudintermedius*, SCC*mec* types II-III, III, IV, V, and VII and some non-typable cassettes have been observed (48), of which, SCC*mec* II-III, SCC*mec* V, and SCC*mec* VII-241 have been sequenced (36).

Interestingly, SCC*mec* II-III contains elements of the SCC*mec* II from *S. epidermidis* and SCC*mec* III from *S. aureus* (36). SCC*mec* typing, along with sequence typing, polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP), multilocus sequence typing (MLST) and staphylococcal protein A or *spa* typing, can be used to differentiate strains of *S. pseudintermedius* and are used for epidemiological surveillance (48). As in MRSA, different strains of MRSP predominate in North America than in Europe (48). There is less genetic variability in MRSP than there is in methicillin-susceptible *S. pseudintermedius* which suggests clonal spread of specific genetic types of MRSP. In European MRSP, MLST ST71-*spa* t02-SCC*mec* II-III predominates, whereas, in North America, MLST ST 68-*spa* t06- SCC*mec* type V predominates (48). This being said, there has been evidence of worldwide dissemination of certain MRSP clones (48).

Zoonotic Potential of *Staphylococcus pseudintermedius*

Both healthy animals and people are capable of carrying MRSA or MRSP and may then serve as a reservoir for further dissemination. However, in the context of veterinary medicine, MRSA strains that infect household pets are thought to be of human origin; whereas, MRSP strains are thought to originate from an animal reservoir (48, 50). Humans are not normally carriers of *S. pseudintermedius*; however, humans in close contact with animals such as dog owners or people in the veterinary profession are at increased risk for colonization or infection with MRSP (41). As mentioned previously, *S. pseudintermedius* can occasionally infect humans, but just as concerning, it can

sometimes colonize humans as well. In a study by van Duijkeren et al., animals in contact with a MRSP-infected animal were also frequently culture-positive; whereas, humans living in the household or working in the veterinary clinic where those animals were treated were positive less frequently (49). Similarly, in their longitudinal study of MRSP, Laarhoven et al. discovered that in addition to the animal infected with MRSP, contact animals, owners, and the environment, both where the infected animal had access and where it did not have access, all were capable of producing positive swabs for MRSP in some of the households (24). In the same study, it was shown that animals infected with MRSP were capable of both long-term colonization (during the entire six month study, with one dog also having a positive sample one year after the initial sample) and intermittent colonization with up to three months between positive samples (24). In a similar study by Windahl et al., 61% of dogs were MRSP-positive for at least 8 months, although they did not test household contacts and could not rule out the possibility of reinfection from such sources (51). Carriage of MRSP in animals and humans could potentially lead to transfer of antimicrobial resistance from the transient colonizer to the normal host commensal bacteria. De Lucia et al. reported the prevalence of MRSP among SIG isolates over a 2 month period at a referral veterinary lab in Italy to be 21%, much higher than the 7.4% previously reported for dogs in Germany (8). Combining these data with the fact that staphylococci are able to transfer drug resistance elements between each other raises great concern. With increasing resistance to multiple classes of antimicrobial drugs in both *S. aureus* (26) and *S. pseudintermedius* (14, 36, 48, 50), doctors and veterinarians have begun using mupirocin to eliminate these organisms.

Mupirocin

Mupirocin is a bacteriostatic antimicrobial that reversibly binds bacterial isoleucyl tRNA synthetase, thereby preventing protein synthesis in most Gram-positive and some Gram-negative bacteria. The naturally occurring form of mupirocin, pseudomonic acid A, is produced by *Pseudomonas fluorescens* and is inhibitory to Gram-positive bacteria and the Gram-negative bacteria *Neisseria gonorrhoeae* and *Haemophilus influenzae* (2, 15). Mupirocin has a high affinity for protein binding and has a distinct reduction in antimicrobial activity in the presence of serum and is therefore limited to topical use clinically (15). Mupirocin, with minimum inhibitory concentrations (MICs) for most bacteria between 0.01 to 0.05 µg/ml, is most notable for its efficacy in elimination of both methicillin-sensitive *Staphylococcus aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) in nasal carriers in hospital settings (2). This is its primary clinical use in humans. Mupirocin has also been approved by the U.S. Food and Drug Administration for use as a topical antimicrobial in dogs with pyoderma. The most common veterinary use of mupirocin is for topical therapy of MRSP associated with canine pyoderma or skin wounds. It is notable that antimicrobial use has not been proven to be effective in decolonizing animals carrying MRSP (48). The use of mupirocin to eliminate nasal carriage of MRSA in humans has made resistance to mupirocin concerning.

Mupirocin Resistance

Two levels of mupirocin resistance occur in staphylococci: Low-level resistance and high-level resistance. Low-level mupirocin resistance (MIC between 8 µg/mL and 256 µg/mL) arises due to point mutations to the chromosomal *ileS* gene, which encodes the native isoleucyl tRNA synthetase (25). Conversely, high-level mupirocin resistance (MIC ≥ 512 µg/mL) occurs through the acquisition of the plasmid-borne *ileS2* gene, which encodes an alternate synthetase to which mupirocin does not bind (25); although, a chromosomal location of *ileS2* has been previously reported (38). *Pseudomonas aeruginosa* has also been found to be resistant to mupirocin (MIC > 1,024 µg/mL) (22). Caffrey et al. conducted a study in 2010 to identify risk factors associated with the development of mupirocin resistance in *S. aureus*; subsequently, they found that infection with mupirocin-resistant MRSA was more common in patients who had undergone surgical procedures, had chronic skin ulcers or had been infected with *P. aeruginosa* during the year prior to culture than in patients infected with mupirocin-susceptible *S. aureus* (5). Because antimicrobial use increases the risk for selection of resistant bacteria, antibiotic therapy should be thoroughly considered prior to use. Summarizing the principles of the European Wound Management Association's document on the management of human wound infections, the goals in wound management are to optimize environmental factors to promote wound healing, to only use antibiotics when specifically indicated, and to choose antimicrobial therapies accordingly, to reduce the chances of selection for antimicrobial-resistant strains of bacteria (13).

Mupirocin Resistance in *Staphylococcus pseudintermedius*

Mupirocin has been used on only a limited basis in veterinary medicine, primarily to treat pyoderma in dogs (9). *S. pseudintermedius* is a common cause of skin infections, otitis externa, and post-operative infections in dogs. As in *S. aureus*, multi-drug resistance is emerging in *S. pseudintermedius*, and, methicillin-resistant *S. pseudintermedius* (MRSP) is an increasing problem (48). In a study conducted by Penna and colleagues in Brazil, 37.1% of *S. pseudintermedius* isolates cultured from cases of otitis externa in dogs were resistant to mupirocin (34). Furthermore, Hurdle et al. discovered that mupirocin resistance could be passed from *S. epidermidis* and MRSA to *S. aureus* RN2677 *in vivo* (20). It has also been found that mupirocin and tetracycline resistance have been conferred by the same plasmid in *S. aureus* (33). Because staphylococci are capable of transferring plasmids between species, it is conceivable that *S. pseudintermedius* possessing plasmids carrying a mupirocin resistance gene may be able to transfer the plasmid or resistance gene to *S. aureus* in human carriers. This could have significant consequences for patients colonized with MRSA or patients with wounds caused by MRSA for which mupirocin ointment would be a potential therapy.

Mupirocin Resistance Plasmids

Mupirocin resistance plasmids appear to be a modified pG01 plasmid, a well-described conjugative plasmid in staphylococci that carries resistance genes for resistance to aminoglycosides, trimethoprim, and quaternary ammonium compounds (32). Plasmid

pG01 can be transferred from one species of *Staphylococcus* to another and contains nine copies of an insertion sequence (IS)-like element, IS431-IS257, of which eight are directly repeated (32). From their data, Morton et al. proposed the manner by which mupirocin resistance plasmids have evolved. They concluded that the smallest mupirocin resistance plasmid, pG0400, was created via recombination of the intervening DNA between the IS repeats on pG01; whereby, alternate antimicrobial resistance genes and an integrated copy of a smaller plasmid, pUB110, were deleted when the mupirocin resistance gene was integrated into the IS elements (32). Additionally, Udo et al. experimentally demonstrated that mupirocin-resistant conjugative plasmids are able to mobilize non-conjugative plasmids, similar to staphylococcal gentamicin resistance plasmids (45).

Based on the premise that mupirocin resistance plasmids have a pSK41/pGO1 backbone with the *ileS2* gene inserted within the IS257 repeats, Perez-Roth et al. created a novel system for classifying plasmids according to the PCR amplification pattern of their IS257-*ileS2* junctions that categorized plasmids into 15 structural groups (35). Perez-Roth et al. concluded that using this method, combined with molecular typing techniques, future spread of mupirocin resistance could be monitored and controlled more efficiently (35). Recently *ileS2* plasmid-mediated mupirocin resistance was found in a mupirocin-resistant, methicillin-susceptible *S. pseudintermedius* isolate in Croatia (30). Using the same PCR amplification methods, Matanovic and colleagues sequenced the IS257-*ileS2* junctions of that *S. pseudintermedius* mupirocin resistance plasmid and discovered a novel arrangement (30).

Summary

In summary, *S. pseudintermedius* is the primary pathogenic staphylococci in dogs and can be associated with infections in cats. Because mupirocin is used in elimination of human carriage of MRSA and for treatment of canine pyoderma, mupirocin resistance is of great concern. The primary goals of this study were to determine the prevalence of mupirocin resistance in *S. pseudintermedius* isolates from dogs in Texas, to classify the level mupirocin resistance, and to assess the molecular structure of the mupirocin resistance genes isolated.

CHAPTER II

PRESENT STUDY

Synopsis

Use of mupirocin in veterinary medicine is primarily limited to the treatment of canine pyoderma caused by methicillin-resistant *S. pseudintermedius* (MRSP). Only one isolate of 572 *S. pseudintermedius* isolates tested was resistant to mupirocin and carried the high-level mupirocin resistance gene, *ileS2*.

Introduction

Staphylococcus pseudintermedius is the primary bacterial pathogen isolated from canine skin lesions, such as those found in pyoderma, and also causes post-surgical infections in dogs (14, 16). Methicillin resistance and multi-drug resistance are increasing in *S. pseudintermedius* thus limiting the options for therapeutic treatment of canine skin infections (48). Mupirocin is a bacteriostatic antibiotic that reversibly binds to isoleucyl tRNA synthetase to disrupt protein synthesis and is widely used to eliminate nasal carriage of methicillin-resistant *Staphylococcus aureus* (MRSA) in human MRSA carriers. Mupirocin has a high affinity for protein binding and has a reduced effectiveness in human serum; therefore, mupirocin is only approved for topical use (2). Mupirocin has been used on only a limited basis in veterinary medicine, primarily to treat pyoderma in dogs caused by methicillin-resistant *S. pseudintermedius* (MRSP) (9).

In *S. aureus*, two levels of mupirocin resistance have been identified. Low-level mupirocin resistance occurs due to a point mutation to the chromosomal *ileS* gene that encodes the native isoleucyl-tRNA synthetase and the minimum inhibitory concentration (MIC) for mupirocin for staphylococci carrying the low-level resistance ranges from 8 µg/mL to 256 µg/mL. Conversely, high-level resistance (MIC ≥ 512 µg/mL) is usually conferred by the plasmid-borne *ileS2*, although a chromosomal location of *ileS2* has been reported (38). Recently, *ileS2* plasmid-mediated mupirocin resistance was found in a mupirocin-resistant, methicillin-susceptible *S. pseudintermedius* isolate in Croatia (30). The goal of the present study was to determine the prevalence of mupirocin resistance in *S. pseudintermedius* isolates in Texas and to characterize the genes involved.

Materials and Methods

In this study, 572 isolates of *S. pseudintermedius* were screened for phenotypic low-level mupirocin resistance. Isolates were collected from veterinary patients, predominantly dogs (*n* = 447), but also included isolates from cats (*n* = 9). Some animals were cultured multiple times in the course of their treatment or were cultured at multiple sites (e.g. nares and perineum). Sources of isolates included a historical collection of 202 isolates from 159 clinical canine infections and 6 feline infections, and contained both MRSP (*n* = 75) and methicillin-susceptible *S. pseudintermedius* (MSSP) (*n* = 127). An additional 195 isolates of MRSP (*n* = 58) and MSSP (*n* = 137) were collected from 162 clinical canine and 3 feline infections between September 22, 2010 and February 8, 2012 concurrent with a study of MRSP prevalence in patients without clinical staphylococcal

infection that presented for elective orthopedic procedures. The prevalence study yielded 175 isolates of MRSP (*n* = 13) and MSSP (*n* = 162) collected from the nares or perineum of 126 dogs (Table 1). The isolates from clinical infections were collected from the following anatomic sites skin (*n* = 95), external ear canal (*n* = 40), wounds (*n* = 53), post-operative infections (*n* = 42), and urine/urinary tract (*n* = 88), respiratory tract (*n* = 16), reproductive tract (*n* = 9) and other sources (*n* = 54) (Table 2).

All isolates were presumptively identified as *S. pseudintermedius* at the time of collection by Gram-stain reaction, colony color, and biochemical tests including the ability to produce hemolysis on trypticase soy agar supplemented with 5% sheep blood agar (BD Diagnostic Systems, USA), to produce coagulase, to produce catalase, and to grow on salt-mannitol agar and based on their susceptibility to polymyxin B. At the time of initial collection, isolates were tested for antimicrobial susceptibility using commercially available systems (GPS card, VITEK, bioMérieux, France; COMPAN1F and COMPAN2F panels, TREK Sensititre, TREK Diagnostics, USA) and tested for methicillin resistance by oxacillin disk diffusion testing and polymerase chain reaction for the presence of *mecA*. Isolates were frozen in 10% glycerol at -80°C in 96-well deep well plates and later inoculated aseptically using a 96-pin replicator onto BBL TSA II 5% sheep's blood agar plates as a control and onto Mueller-Hinton (MH) agar (BD Diagnostic Systems) supplemented with 8 µg/ml mupirocin (Sigma-Aldrich; USA) agar plate (mupirocin plate hereafter) to screen for low-level resistance to mupirocin. *Pseudomonas aeruginosa* (ATCC 27853) was used as a positive control for mupirocin resistance as it was shown to be mupirocin resistant (22). Plates were incubated for 24 hours at 37°C.

Plasmid purification was accomplished using the QIAprep Mini Spin kit plasmid purification kit (QIAgen, Valencia, CA). The mupirocin-resistant colony was struck onto a mupirocin plate and incubated overnight at 37°C. A single colony was then aseptically transferred to 5 ml of Luria-Bertani broth (LB) and incubated on a C24 Innova shaking incubator (New Brunswick, USA) at 220 RPM at 37°C for 15 hours. Using 2 aliquots of 2 ml of sample each, the manufacturer's instructions were followed, combining the aliquots during resuspension of the pellets of cells. In the final step, DNA was eluted using nuclease-free water. Samples were then tested for concentration and quality using a NanoDrop® spectrometer (Thermo Scientific, USA) prior to downstream reactions.

To perform polymerase chain reaction (PCR) for identification of the high-level resistance gene *ileS2*, the previously published primers mupA and mupB (2) and primers M1 and M2 (25) were used to amplify 458 bp and 237 bp fragments, respectively, of the *ileS*2 gene (Table 3). A total reaction volume of 50 µl was established for each set of primers as a separate reaction using 30.5 µl sterile distilled water, 5 µl 10X buffer, 5 µl $MgCl_2$, 4 µl dNTPs, 2.5 µl of the 5' primer and 2.5 µl of the 3' primer and 0.5 µl Taq polymerase per reaction (Lucigen Corp., USA). Three to five colonies were isolated from the mupirocin plate and suspended into the 50 µl reaction. Reactions were run in thermal cycler using the settings: 95°C for 5 min., thirty-five cycles of 95°C for 30 sec., 57°C for 30 sec., and 72°C for 30 sec., then 72°C for 7 min., and then held at 4°C. Negative controls used were water with no template DNA and a known mupirocin-sensitive, methicillin-susceptible *S. aureus*, ATCC strain 29213. No positive controls were available; therefore, two different segments of the gene were chosen to identify the presence of *ileS2*. The products were then run on a 2% agarose gel

for 2 hours at 70V, visualized with GelRed (Phenix Research, USA) and compared to a 100 bp molecular weight marker (Invitrogen, USA).

To determine the structural type of the plasmid carrying the *ileS2* gene, PCR was run using the previously published primers: $IS257_F$, *ileS2*-5', *ileS2*-3', and $IS257_R$ (Table 3) in various combinations, under the conditions: 94°C for 5 min., thirty cycles of 94°C for 30 sec., 60°C for 40 sec., and 72°C for 60 sec., then 72°C for 10 min., and then held at 4°C (35). PCR products from colony-PCR using the mupirocin-resistant strain, purified plasmid, and negative controls of colony PCR of ATCC strain 29213 and water with no template DNA were then run on 1% agarose gel at 80V for 90 min., visualized with GelRed and compared to the 1 kb Plus molecular weight marker (Invitrogen, USA). An additional PCR amplification was necessary to obtain an internal fragment of the upstream junction of *ileS2*, using novel primers *ileS* 518F and *ileS* 1186R (Table 3).

PCR was performed to identify the native *ileS* gene using the primers *ileS*-F1 and *ileS*-R1 (30) (Table 3). Conditions for thermal cycler were: 94°C for 5 min.; 30 cycles of 94°C for 30 sec., 55°C for 30 sec., 72°C for 60 sec., then 72°C for 7 min., and held at 4°C.

PCR products were purified using either the QIAprep Gel Purification kit (QIAgen, Valencia, CA) or the Zymoclean Gel DNA Recovery kit (Zymo Research; Irvine, CA) according to the manufacturers' protocols. Purified PCR products were then cloned into the pT7Blue plasmid vector using the Novagen pT7Blue Perfectly Blunt Cloning Kit (EMD Chemicals, Inc.; Darmstadt, Germany) following manufacturer's protocol. Resultant plasmids containing the upstream IS257-*ileS2* junction, the downstream *ileS2*-IS257 junction and the fragment of the native *ileS* gene were submitted

to the Texas A&M Gene Technologies Lab for sequencing. Resultant sequences were compared to sequences JX186508, JX186509, JX186511, JX186512, JX186513, and JX186514 (30) and, HQ625435, HQ625436, HQ625437, and HQ625438 (35) in GenBank and using MEGA5.1 software (44).

Results

Of the 572 isolates tested, only one isolate, 39-045, was resistant to mupirocin by testing on mupirocin agar (Fig. 1). Isolate 39-045 was originally cultured from the nares of a healthy, one-year-old, castrated, male, Bernese mountain dog presenting to the Texas A&M College of Veterinary Medicine for an orthopedic evaluation. This isolate was pan-susceptible to all antimicrobials tested using the COMPAN2F drug panel and negative for the presence of the *mecA* gene via PCR analysis. Of the 175 isolates collected from healthy dogs that presented for elective orthopedic procedures, the prevalence of mupirocin resistance was 1 in 126 dogs or 0. 8%. During this study, 195 *S. pseudintermedius* isolates were collected from 162 dogs and 3 cats with clinical infections resulting in a total of 370 *S. pseudintermedius* isolates from 288 dogs from September 22, 2010 and February 8, 2012 with an overall prevalence of 1 in 288 (0.3%). The prevalence of mupirocin-resistant *S. pseudintermedius* from the historical collection of 202 isolates from 159 dogs and 6 cats was 0 out of 159 dogs.

Upon PCR analysis using primers mupA and mupB, isolate 39-045 contained a fragment between 300 and 400 bp (Fig. 2). Similarly, PCR analysis with M1 and M2 primers revealed a band between 200 and 300 bp (Fig 2).

Next, isolate 39-045 was evaluated using PCR for the IS257-*ileS2* junctions using primers *ileS2*-5', $IS257_F$, *ileS2*-3', and $IS257_R$. Isolate 39-045 contained a band between 1650 and 2000 bp for primers *ileS2*-5' and $IS257_R$ and a band between 1000 and 1650 bp for primers *ileS2*-3' and $IS257_F$ (Fig. 3). These bands were then sequenced and compared to previously published sequences. Sequence analysis, using NCBI BLAST analysis, indicated a 100% similarity between isolate 39-045 and the previously published *ileS2* sequences from *S. pseudintermedius* JX186508 and a 97% similarity between 39-045 and JX186509 (30) and a 99% similarity between isolate 39-045 and the previously published sequence from *S. aureus* structural group S2 *ileS2* plasmid HQ625436 (35).

PCR analysis for the *ileS* gene was performed using the previously published primers *ileS*-F1 and *ileS*-R1 (30). PCR analysis revealed a band between 850 and 1000 bp (Fig. 4). This fragment was also sequenced and compared to previously published sequences using MEGA5.1 software. NCBI BLAST analysis indicated a 99% similarity between isolate 39-045 and the previously published sequences of the *S. pseudintermedius* chromosomal *ileS* gene: JX186511, JX186512, JX186513, JX186514 (30). Using MEGA5.1 software, three point mutations were identified between the native *ileS* of isolate 39-045 and that of *S. pseudintermedius* ED99 (NC_017568) (53) from T to C in our sequence at position 2082, T to C at 2097, and G to A at 2121. Similarly, compared to the Matanovic sequence JX186511, there were four point mutations: From T to C mutation in our sequence at position 1413, A to G at 2076, T to C at 2082 and G to A at 2121.

Discussion

Screening of the 572 *S. pseudintermedius* isolates for low-level mupirocin resistance revealed that only one isolate was resistant to mupirocin. Following phenotypic testing for low-level resistance, the isolate was analyzed for the presence of high-level mupirocin resistance by PCR amplification of two different regions of the plasmid-borne *ileS2* gene. The presence of bands at approximately 458-bp with mupA and mupB primers and approximately 237-bp with M1 and M2 primers indicate that isolate 39-045 contains the *ileS2* gene (Fig. 2). To further determine structural type of the plasmid, PCR for the IS257-*ileS2* spacer regions was performed following a previously published molecular classification system (35). The fragments are consistent with the amplification for structural group S2 *ileS2* plasmids found in *S. aureus*, pattern II, with bands sized 1127-bp for primers *ileS2*-3' & $IS257_F$ and at 1816-bp for primers $IS257_R$ & *ileS2*-5'(Figure 3).

Previous work with the IS257-*ileS2* junctions has been done with *S. aureus* (35, 52) and in *Staphylococcus haemolyticus* (11). Recently, plasmid-borne *ileS2* was identified in *S. pseudintermedius* isolated from a dog with pyoderma in Croatia (30). Sequence analysis indicated a 100% similarity between isolate 39-045 and the previously published *ileS2* sequences from *S. pseudintermedius*, JX186508 and a 97% similarity between 39-045 and JX186509 (30) and a 99% similarity between isolate 39-045 and the previously published sequence from *S. aureus* structural group S2 *ileS2* plasmid HQ625436 (35), supporting the concept that *S. aureus* and *S. pseudintermedius* share similar plasmids.

To determine whether isolate 39-045 carried a concurrent *ileS* mutation, PCR amplification of the chromosomal *ileS* gene was also performed using previously published primers *ileS*-F1 and *ileS*-R1 (30) (Fig 4). The resultant 956 bp product was sequenced and analyzed using MEGA5.1 software, and analysis indicated a 99% similarity between isolate 39-045 and the previously published sequences of the *S. pseudintermedius* chromosomal *ileS* gene: JX186511, JX186512, JX186513, JX186514 (30). There were three point mutations from the native *ileS* of *S. pseudintermedius* ED99 (NC_017568) from T to C in our sequence at position 2082, T to C at 2097, and G to A at 2121. Likewise, compared to the Matanovic sequence JX186511, there were four point mutations: From T to C mutation in our sequence at position 1413, A to G at 2076, T to C at 2082 and G to A at 2121. All point mutations were silent, causing no change in the amino acid sequence.

In summary, we found that the prevalence of mupirocin resistance was 0.8% (1/126) in healthy dogs without active, clinical staphylococcal infections. While no mupirocin resistant isolates were found in our collection of isolates from dogs with clinical disease, the presence of plasmid-mediated mupirocin resistance is of concern as previous work has demonstrated that mupirocin resistance can be transmitted from one species of *Staphylococcus* to another in vivo (20). Increased rates of methicillin-resistance and multi-drug resistance in *S. pseudintermedius* and approval of mupirocin for use in dogs have made mupirocin an attractive alternative for topical use in canine pyoderma (48). This could result in increased mupirocin-resistance in *S. pseudintermedius* over time. With 36.5 percent of U.S. households owning a dog in 2012 (3), there is a great potential for transmission of mupirocin resistance from canine isolates

of *S. pseudintermedius* to human isolates of *S. aureus* or vice versa. This could have significant public health implications. For these reasons, mupirocin resistance should be monitored and mupirocin use should be thoroughly considered before prescribing to canine patients.

CHAPTER III

DISCUSSION AND CONCLUSION

In this study, 572 samples of *S. pseudintermedius*, isolated from patients, mostly dogs, of the Texas A&M University College of Veterinary Medicine veterinary hospital, were analyzed for their susceptibility to mupirocin. Of the 572 isolates, only one was phenotypically resistant to a low-level of mupirocin (8 µg/ml). Subsequently, this isolate was analyzed by PCR and sequence analysis to determine whether it contained mutations in the chromosomal *ileS* gene or carried the high-level mupirocin resistance gene, *ileS2* on a plasmid. PCR testing with primers that target *ileS2* demonstrated the presence of bands at approximately 458 bp with primers mupA and mupB and at around 237 bp with primers M1 and M2, indicating the isolate contains the *ileS2* gene. Fifteen structural plasmid types have been identified in *S. aureus* based on orientation and position of the IS257-*ileS2* spacer regions (35). At the time that we initiated this investigation, there were no published accounts of the plasmid structure of mupirocin resistance plasmids in *S. pseudintermedius*; therefore, to further determine structural type of the plasmid, the PCR for the *S. aureus* IS257-*ileS2* junctions was performed. The fragments are consistent with amplification pattern II, with bands at 1127 bp for primers *ileS2*-3' and $IS257_F$ and at 1816 bp for primers $IS257_R$ & *ileS2*-5', which is consistent with structural group S2 *ileS2* plasmids. Previous work with the IS257-*ileS2* junctions has been done with *S. aureus* (35, 52) and in *Staphylococcus haemolyticus* (11). Subsequent to our study, the structure of a mupirocin resistance plasmid from *S. pseudintermedius* from Croatia was published in December 2012 (30) . That study, as well as our investigation,

confirms the same pattern of IS257-*ileS2* junction for S2 plasmids in *S. pseudintermedius* as in *S. aureus*. This information, combined with the knowledge that staphylococci are able to transfer plasmids via conjugation from one species to another, indicates that mupirocin resistance is capable of being transferred from the dog-colonizing *S. pseudintermedius* to the human-colonizing *S. aureus* and vice versa. While we found that mupirocin resistance was uncommon in our patient population the mupirocin-resistant isolate that we found came from a healthy dog that would not have been routinely tested. This could indicate that mupirocin resistance occurs more often than our study would suggest. With 36.5 percent of U.S. households owning a dog in 2012 (3), there is a great potential for transmission of mupirocin resistance from animal strains of staphylococci to human strains or vice versa. In the recent paper describing the mupirocin-resistant *S. pseudintermedius* isolate in Croatia, the owner of the colonized dog was a nurse. We did not ask pet owners any questions with regard to the health of human family members so it is not possible to determine the origin of the mupirocin-resistance plasmid in our isolate. With recent increases in multi-drug resistance in both *S. aureus* and *S. pseudintermedius* (48), mupirocin has been increasingly used to treat resistant forms of both bacteria. For these reasons, mupirocin resistance should be monitored and clinical use of mupirocin must be fully justified before prescribing.

Antimicrobial therapy should be carefully considered in any situation, but especially in cases where multi-drug resistance is likely to occur. In treatment of dogs, bacterial culture and susceptibility testing should be implemented when infections fail to respond to empiric therapy, clinical lesions are consistent with deep pyoderma, there is cytological evidence of a mixed infection, the dog's condition relapses, there has been

recent antimicrobial administration for any reason, or if the dog has been diagnosed with MRSP previously (6). In lieu of antimicrobial therapy for treatment of MRSP, topical use of chlorhexidine- or iodine-containing products to decontaminate the skin and coat and cleaning/disinfection of surfaces within the dog's home may be viable alternative measures for the treatment of MRSP infections in dogs (48). Valentine et al. validated these findings in their study, showing that the clinical concentrations of chlorhexidine used to treat pyoderma are over 3000 times higher than the MIC for both MSSP and MRSP (46). Other therapy options include shampoos or leave-in conditioners containing 10% ethyl lactate or 2.5-3% benzoyl peroxide with potentiating ingredients such as chitosan, liposomes, or lipid barriers that enhance contact time or penetration, depending on polarization of the active ingredient and the charge of the potentiating agent (21). Frequently, combinations of therapies work best. Considerations when choosing topical treatment of pyoderma include location/extent of the infection, hair coat involvement, antibiotic therapy and its means of delivery, and the owner's ability/willingness to comply (21).

Staphylococci are capable of transferring antimicrobial resistance from one species to another. Furthermore, conjugative mupirocin resistance plasmids are able to transfer between coagulase-negative and coagulase-positive staphylococci (32). In a report by Hurdle et al., a patient in a nursing home undergoing mupirocin treatment for persistent MRSA carriage acquired high-level mupirocin resistance through conjugative transfer from a mupirocin-resistant strain of *S. epidermidis* (20). These data support the concept that *S. pseudintermedius* may serve as a reservoir for mupirocin resistance for *S. aureus*, including MRSA.

In conclusion our results show that although mupirocin resistance is not common in *S. pseudintermedius* isolates from animals in Texas, it does occur. This is in keeping with similar studies (28, 30, 36, 46), but the fact that the mupirocin-resistant isolate was cultured from a healthy dog could indicate a higher prevalence in the general population than reported here. Previous studies have demonstrated that mupirocin resistance can be transmitted from one species of *Staphylococcus* to another through conjugation. Our results show a high degree of similarity between mupirocin resistance plasmids from *S. pseudintermedius* and *S. aureus*. This reinforces the notion that *S. pseudintermedius* can serve as a reservoir for mupirocin resistance for *S. aureus* and vice versa. Therefore, mupirocin resistance should be monitored and careful consideration should be employed before prescribing mupirocin for canine patients.

REFERENCES

1. **Abraham, J. L., D. O. Morris, G. C. Griffeth, F. S. Shofer, and S. C. Rankin.** 2007. Surveillance of Healthy Cats and Cats with Inflammatory Skin Disease for Colonization of the Skin by Methicillin-Resistant Coagulase-Positive Staphylococci and *Staphylococcus schleiferi* ssp. *schleiferi*. Veterinary Dermatology **18:**252-259.
2. **Anthony, R. M., A. M. Connor, E. G. M. Power, and G. L. French.** 1999. Use of the Polymerase Chain Reaction for Rapid Detection of High-Level Mupirocin Resistance in Staphylococci. European Journal of Clinical Microbiology & Infectious Diseases **18:**30-34.
3. **AVMA** 2012, posting date. U.S. Pet Ownership Statistics. https://www.avma.org/KB/Resources/Statistics/Pages/Market-research-statistics-US-pet-ownership.aspx. [Online.]
4. **Bannoehr, J., A. Franco, M. Iurescia, A. Battisti, and J. R. Fitzgerald.** 2009. Molecular Diagnostic Identification of *Staphylococcus pseudintermedius*. Journal of Clinical Microbiology **47:**469-471.
5. **Caffrey, A. R., B. J. Quilliam, and K. L. LaPlante.** 2010. Risk Factors Associated with Mupirocin Resistance in Meticillin-Resistant *Staphylococcus aureus*. Journal of Hospital Infection **76:**206-210.
6. **Cain, C. L.** 2013. Antimicrobial Resistance in Staphylococci in Small Animals. Veterinary Clinics of North America: Small Animal Practice **43:**19-40.

7. **Campanile, F., D. Bongiorno, S. Borbone, M. Venditti, M. Giannella, C. Franchi, and S. Stefani.** 2007. Characterization of a Variant of the SCC*mec* Element in a Bloodstream Isolate of *Staphylococcus intermedius*. Microbial Drug Resistance **13:**7-10.

8. **De Lucia, M., A. Moodley, F. Latronico, A. Giordano, M. Caldin, A. Fondati, and L. Guardabassi.** 2011. Prevalence of Canine Methicillin-Resistant *Staphylococcus pseudintermedius* in a Veterinary Diagnostic Laboratory in Italy. Research in Veterinary Science **91:**346-348.

9. **Dechra.** Muricin Ointment 2%, Product Insert, vol. NAC No. 12920021. Dechra Veterinary Products, Overland Park, KS.

10. **Devriese, L. A., M. Vancanneyt, M. Baele, M. Vaneechoutte, E. De Graef, C. Snauwaert, I. Cleenwerck, P. Dawyndt, J. Swings, A. Decostere, and F. Haesebrouck.** 2005. *Staphylococcus pseudintermedius* sp. nov., a Coagulase-Positive Species from Animals. International Journal of Systematic and Evolutionary Microbiology **55:**1569-1573.

11. **do Carmo Ferreira, N., R. P. Schuenck, K. R. N. dos Santos, M. d. C. de Freire Bastos, and M. Giambiagi-deMarval.** 2011. Diversity of Plasmids and Transmission of High-Level Mupirocin *mupA* Resistance Gene in *Staphylococcus haemolyticus*. Federation of European Microbiological Societies Immunology and Medical Microbiology **61:**147-152.

12. **Enright, M. C.** 2008. The Population Structure of *Staphylococcus aureus*, p. 29-39. *In* J. A. Lindsay (ed.), *Staphylococcus*: Molecular Genetics. Caister Academic Press, Norfolk,UK.

13. **EWMA.** 2006. Position Document: Management of Wound Infection, European Wound Management Association. MEP Ltd, London, UK.

14. **Fitzgerald, J. R., and J. R. Penades.** 2008. Staphylococci of Animals, p. 255-263. *In* J. A. Lindsay (ed.), *Staphylococcus*: Molecular Genetics. Caister Academic Press, Norfolk, UK.

15. **Fuchs, P. C., R. N. Jones, and A. L. Barry.** 1990. Interpretive Criteria for Disk Diffusion Susceptibility Testing of Mupirocin, a Topical Antibiotic. Journal of Clinical Microbiology **28:**608-609.

16. **Fulham, K. S., S. L. Lemarie, G. Hosgood, and H. L. N. Dick.** 2011. In Vitro Susceptibility Testing of Meticillin-Resistant and Meticillin-Susceptible Staphylococci to Mupirocin and Novobiocin. Veterinary Dermatology **22:**88-94.

17. **Gerstadt, K., J. S. Daly, M. Mitchell, M. Wessolossky, and S. H. Cheeseman.** 1999. Methicillin-Resistant *Staphylococcus intermedius* Pneumonia Following Coronary Artery Bypass Grafting. Clinical Infectious Diseases **29:**218-219.

18. **Gómez-Sanz, E., C. Torres, C. Lozano, and M. Zarazaga.** 2013. High Diversity of *Staphylococcus aureus* and *Staphylococcus pseudintermedius* Lineages and Toxigenic Traits in Healthy Pet-Owning Household Members. Underestimating Normal Household Contact? Comparative Immunology, Microbiology and Infectious Diseases **36:**83-94.

19. **Guardabassi, L., M. E. Loeber, and A. Jacobson.** 2004. Transmission of Multiple Antimicrobial-Resistant *Staphylococcus intermedius* Between Dogs Affected by Deep Pyoderma and Their Owners. Veterinary Microbiology **98:**23-27.

20. **Hurdle, J. G., A. J. O'Neill, L. Mody, I. Chopra, and S. F. Bradley.** 2005. In Vivo Transfer of High-Level Mupirocin Resistance from *Staphylococcus epidermidis* to Methicillin-Resistant *Staphylococcus aureus* Associated with Failure of Mupirocin Prophylaxis. Journal of Antimicrobial Chemotherapy **56:**1166-1168.

21. **Jeffers, J. G.** 2013. Topical Therapy for Drug-Resistant Pyoderma in Small Animals. Veterinary Clinics of North America: Small Animal Practice **43:**41-50.

22. **Jones, R. N., Q. Li, B. Kohut, D. J. Biedenbach, J. Bell, and J. D. Turnidge.** 2006. Contemporary Antimicrobial Activity of Triple Antibiotic Ointment: A Multiphased Study of Recent Clinical Isolates in the United States and Australia. Diagnostic Microbiology and Infectious Disease **54:**63-71.

23. **Kempker, R., D. Mangalat, T. Kongphet-Tran, and M. Eaton.** 2009. Beware of the Pet Dog: A Case of *Staphylococcus intermedius* Infection. The American Journal of the Medical Sciences **338:**425-427.

24. **Laarhoven, L., P. de Heus, J. van Luijn, B. Duim, J. Wagenaar, and E. van Duijkeren.** 2011. Longitudinal Study on Methicillin-Resistant *Staphylococcus pseudintermedius* in Households. PLoS ONE **6:**1-6.

25. **Lima de Castro Nunes, E., K. R. N. dos Santos, P. J. J. Mondino, M. d. C. de Freire Bastos, and M. Giambiagi-deMarval.** 1999. Detection of *ileS-2* Gene Encoding Mupirocin Resistance in Methicillin-Resistant *Staphylococcus aureus* by Multiplex PCR. Diagnostic Microbiology and Infectious Disease **34:**77-81.

26. **Lindsay, J. A.** 2008. *S. aureus* Evolution: Lineages and Mobile Genetic Elements, p. 45-62. *In* J. A. Lindsay (ed.), *Stahpylococcus*: Molecular Genetics. Caister Academic Press, Norfolk, UK.

27. **Lindsay, J. A.** 2008. Staphylococcus: Molecular Genetics. Caister Academic Press, Norfolk, UK.

28. **Loeffler, A., S. J. Baines, M. S. Toleman, D. Felmingham, S. K. Milsom, E. A. Edwards, and D. H. Lloyd.** 2008. *In Vitro* Activity of Fusidic Acid and Mupirocin Against Coagulase-Positive Staphylococci from Pets. Journal of Antimicrobial Chemotherapy **62:**1301-1304.

29. **Lowy, F. D.** 2003. Antimicrobial Resistance: The Example of *Staphylococcus aureus*. The Journal of Clinical Investigation **111:**1265-1273.

30. **Matanović, K., E. Pérez-Roth, S. Pintarić, and B. Šeol Martinec.** 2013. Molecular Characterization of High-Level Mupirocin Resistance in *Staphylococcus pseudintermedius*. Journal of Clinical Microbiology **51:**1005-1007.

31. **Morris, D. O., R. C. Boston, K. O'Shea, and S. C. Rankin.** 2010. The Prevalence of Carriage of Meticillin-Resistant Staphylococci by Veterinary Dermatology Practice Staff and Their Respective Pets. Veterinary Dermatology **21:**400-407.

32. **Morton, T. M., J. L. Johnston, J. Patterson, and G. L. Archer.** 1995. Characterization of a Conjugative Staphylococcal Mupirocin Resistance Plasmid. Antimicrobial Agents and Chemotherapy **39:**1272-1280.

33. **Needham, C., M. Rahman, K. G. H. Dyke, and W. C. Noble.** 1994. An Investigation of Plasmids from *Staphylococcus aureus* that Mediate Resistance to Mupirocin and Tetracycline. Microbiology **140:**2577-2583.

34. **Penna, B., R. Varges, L. Medeiros, G. M. Martins, R. R. Martins, and W. Lilenbaum.** 2010. Species Distribution and Antimicrobial Susceptibility of Staphylococci Isolated from Canine Otitis Externa. Veterinary Dermatology **21:**292-296.

35. **Pérez-Roth, E., E. Armas-González, J. Alcoba-Flórez, and S. Méndez-Álvarez.** 2011. PCR-Based Amplification of Heterogeneous IS257-*ileS2* Junctions for Molecular Monitoring of High-Level Mupirocin Resistance in Staphylococci. Journal of Antimicrobial Chemotherapy **66:**471-475.

36. **Perreten, V., K. Kadlec, S. Schwarz, U. Grönlund Andersson, M. Finn, C. Greko, A. Moodley, S. A. Kania, L. A. Frank, D. A. Bemis, A. Franco, M. Iurescia, A. Battisti, B. Duim, J. A. Wagenaar, E. van Duijkeren, J. S. Weese, J. R. Fitzgerald, A. Rossano, and L. Guardabassi.** 2010. Clonal Spread of Methicillin-Resistant *Staphylococcus pseudintermedius* in Europe and North America: An International Multicentre Study. Journal of Antimicrobial Chemotherapy **65:**1145-1154.

37. **Queck, S. Y., and M. Otto.** 2008. *Staphylococcus epidermidis* and Other Coagulase-Negative Staphylococci, p. 227-244. *In* J. A. Lindsay (ed.), *Staphylococcus*: Molecular Genetics. Caister Academic Press, Norfolk, UK.

38. **Ramsey, M. A., S. F. Bradley, C. A. Kauffman, and T. M. Morton.** 1996. Identification of Chromosomal Location of *mupA* Gene, Encoding Low-Level

Mupirocin Resistance in Staphylococcal Isolates. Antimicrobial Agents and Chemotherapy **40:**2820-2823.

39. **Sasaki, T., K. Kikuchi, Y. Tanaka, N. Takahashi, S. Kamata, and K. Hiramatsu.** 2007. Reclassification of Phenotypically Identified *Staphylococcus intermedius* Strains. Journal of Clinical Microbiology **45:**2770-2778.

40. **Sasaki, T., S. Tsubakishita, Y. Tanaka, A. Sakusabe, M. Ohtsuka, S. Hirotaki, T. Kawakami, T. Fukata, and K. Hiramatsu.** 2010. Multiplex-PCR Method for Species Identification of Coagulase-Positive Staphylococci. Journal of Clinical Microbiology **48:**765-769.

41. **Soedarmanto, I., T. Kanbar, H. Ülbegi-Mohyla, M. Hijazin, J. Alber, C. Lämmler, Ö. Akineden, R. Weiss, A. Moritz, and M. Zschöck.** 2011. Genetic Relatedness of Methicillin-Resistant *Staphylococcus pseudintermedius* (MRSP) Isolated From a Dog and the Dog Owner. Research in Veterinary Science **91:**25-27.

42. **Stegmann, R., A. Burnens, C. A. Maranta, and V. Perreten.** 2010. Human Infection Associated with Methicillin-Resistant *Staphylococcus pseudintermedius* ST71. Journal of Antimicrobial Chemotherapy **65:**2047-2048.

43. **Talan, D. A., E. Goldstein, D. Staatz, and G. D. Overturf.** 1989. *Staphylococcus intermedius*: Clinical Presentation of a New Human Dog Bite Pathogen. Annals of Emergency Medicine **18:**410-413.

44. **Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei, and S. Kumar.** 2011. MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum

Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Molecular Biology and Evolution **28:**2731-2739.

45. **Udo, E. E., L. E. Jacob, and B. Mathew.** 2001. Genetic Analysis of Methicillin-Resistant *Staphylococcus aureus* Expressing High- and Low-Level Mupirocin Resistance. Journal of Medical Microbiology **50:**909-915.

46. **Valentine, B. K., W. Dew, A. Yu, and J. S. Weese.** 2012. *In Vitro* Evaluation of Topical Biocide and Antimicrobial Susceptibility of *Staphylococcus pseudintermedius* from Dogs. Veterinary Dermatology **23:**493-495.

47. **Valvatne, H., M. I. A. Rijnders, A. Budimir, M.-L. Boumans, A. J. de Neeling, P. S. Beisser, E. E. Stobberingh, and R. H. Deurenberg.** 2009. A Rapid, 2-Well, Multiplex Real-Time Polymerase Chain Reaction Assay for the Detection of SCC*mec* Types I to V in Methicillin-Resistant *Staphylococcus aureus*. Diagnostic Microbiology and Infectious Disease **65:**384-391.

48. **van Duijkeren, E., B. Catry, C. Greko, M. A. Moreno, M. C. Pomba, S. Pyörälä, M. Ružauskas, P. Sanders, E. J. Threlfall, J. Torren-Edo, and K. Törneke.** 2011. Review on Methicillin-Resistant *Staphylococcus pseudintermedius*. Journal of Antimicrobial Chemotherapy **66:**2705-2714.

49. **van Duijkeren, E., M. Kamphuis, I. C. van der Mije, L. M. Laarhoven, B. Duim, J. A. Wagenaar, and D. J. Houwers.** 2011. Transmission of Methicillin-Resistant *Staphylococcus pseudintermedius* Between Infected Dogs and Cats and Contact Pets, Humans and the Environment in Households and Veterinary Clinics. Veterinary Microbiology **150:**338-343.

50. **Weese, J. S., and E. van Duijkeren.** 2010. Methicillin-Resistant *Staphylococcus aureus* and *Staphylococcus pseudintermedius* in Veterinary Medicine. Veterinary Microbiology **140:**418-429.

51. **Windahl, U., E. Reimegard, B. S. Holst, A. Egenvall, L. Fernstrom, M. Fredriksson, G. Trowald-Wigh, and U. G. Andersson.** 2012. Carriage of Methicillin-Resistant *Staphylococcus pseudintermedius* in Dogs: A Longitudinal Study. BioMed Central Veterinary Research **8:**34-42.

52. **Woodford, N., A. P. Watson, S. Patel, M. Jevon, D. J. Waghorn, and B. D. Cookson.** 1998. Heterogeneous Location of the *mupA* High-Level Mupirocin Resistance Gene in *Staphylococcus aureus*. Journal of Medical Microbiology **47:**829-835.

53. **Zakour, N. L. B., J. Bannoehr, A. H. M. van den Broek, K. L. Thoday, and J. R. Fitzgerald.** 2011. Complete Genome Sequence of the Canine Pathogen *Staphylococcus pseudintermedius*. Journal of Bacteriology **193:**2363-2364.

54. **Zubeir, I. E. M. E., T. Kanbar, J. Alber, C. Lämmler, Ö. Akineden, R. Weiss, and M. Zschöck.** 2007. Phenotypic and Genotypic Characteristics of Methicillin/Oxacillin-Resistant *Staphylococcus intermedius* Isolated from Clinical Specimens During Routine Veterinary Microbiological Examinations. Veterinary Microbiology **121:**170-176.

APPENDIX A

FIGURES

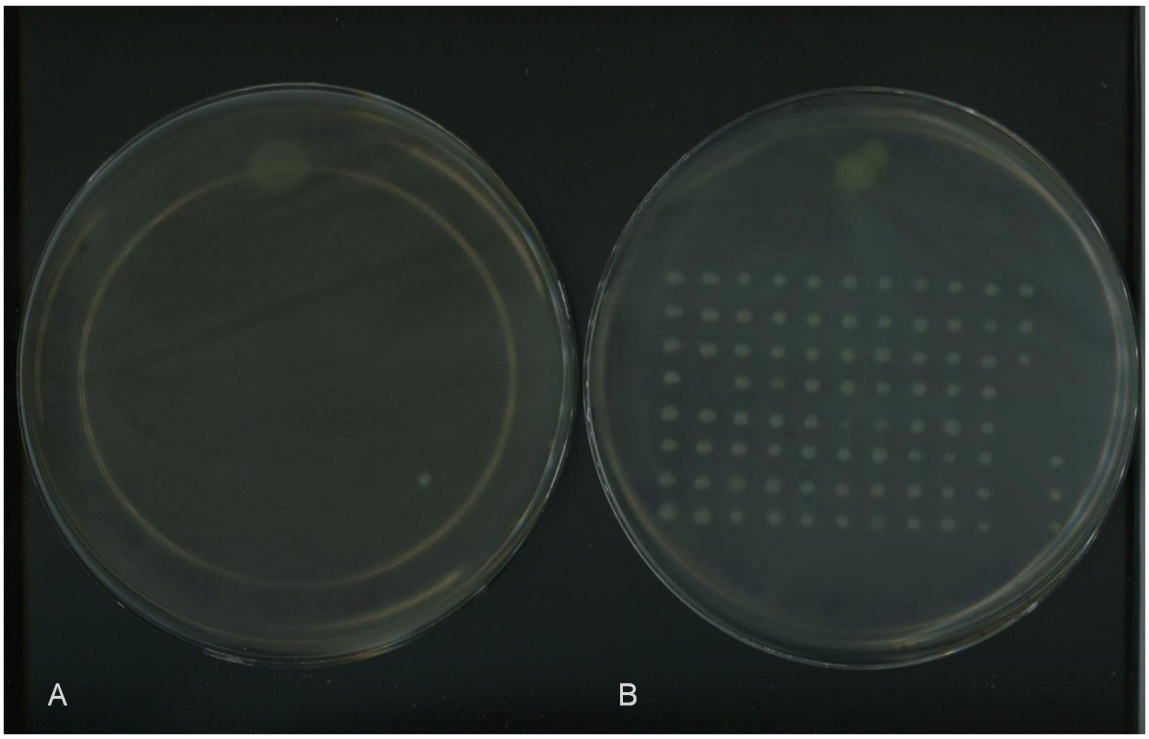

FIG. 1. Mueller-Hinton agar plates demonstrating bacterial growth. Plate (A) supplemented with 8 μg/mL mupirocin shows growth of only isolate 39-045 and the positive control, *P. aeruginosa*; and, an un-supplemented MH agar plate (B), demonstrates growth of all isolates with the exception of the isolate at position D2 within the deep well plate. This isolate was restruck on both a mupirocin agar plate and a control plate on a later date to screen for resistance.

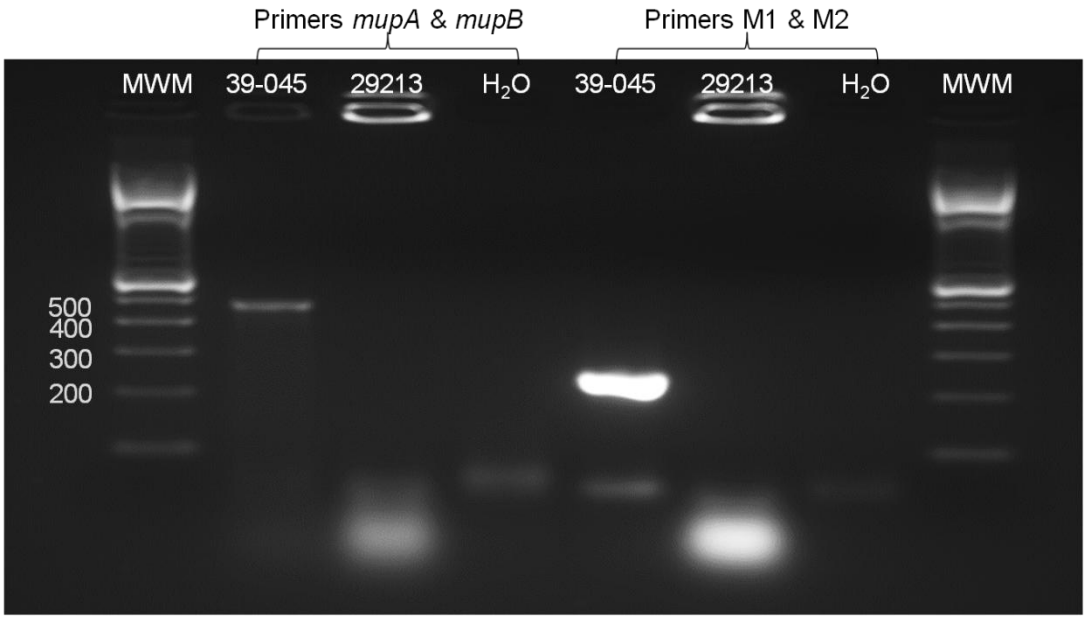

FIG. 2. Presence of *ileS2* in *Staphylococcus pseudintermedius* from a dog. Agarose gel electrophoresis patterns showing PCR amplification products for *ileS2* using primers mupA and mupB and primers M1 and M2. MWM indicates 100 bp molecular weight marker, 39-045 indicates methicillin-susceptible, mupirocin-resistant *S. pseudintermedius* isolate from a canine patient, 29213 indicates the methicillin-susceptible, mupirocin-susceptible, *S. aureus* ATCC 29213 used as a negative control, and H_2O indicates water with no template DNA, also used as a negative control. No positive controls were available.

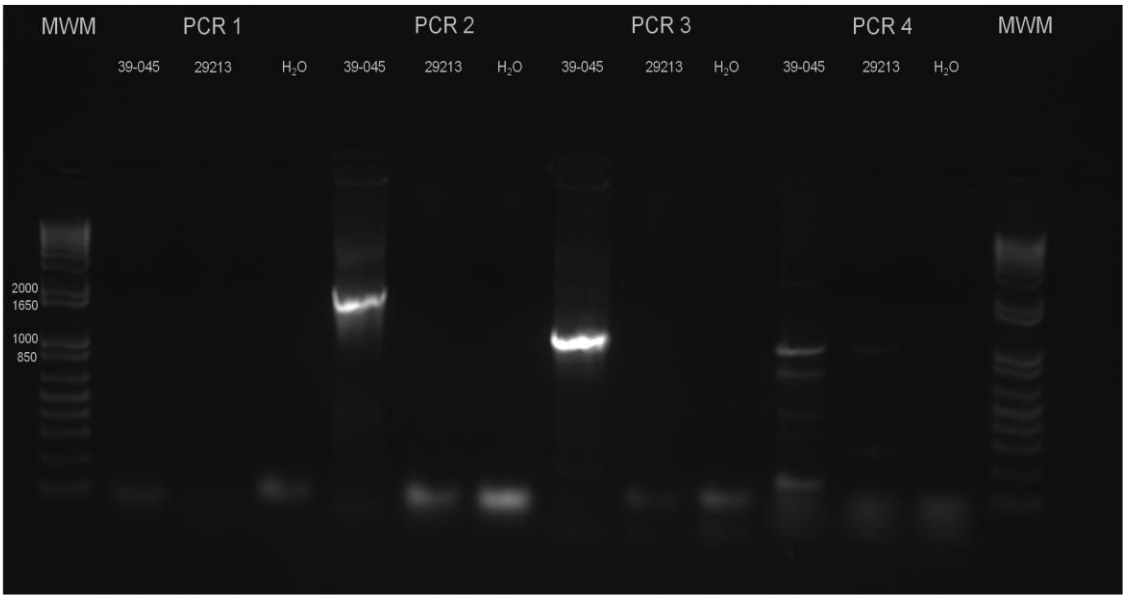

FIG. 3. Agarose gel electrophoresis patterns of products from PCR amplification of the IS257-*ileS2* junctions of isolate 39-045. Primer pairs for each reaction were as follows: PCR 1 - *ileS2*-5' and $IS257_F$; PCR 2 - *ileS2*-5' and $IS257_R$; PCR 3 - *ileS2*-3' and $IS257_F$; and PCR 4 - *ileS2*-3' and $IS257_R$. MWM indicates 1 kb molecular weight marker, 39-045 indicates methicillin-susceptible, mupirocin-resistant *S. pseudintermedius* isolate, 29213 indicates the methicillin-susceptible, mupirocin-susceptible *S. aureus* ATCC 29213 used as a negative control, and H_2O indicates water with no template DNA, also used as a negative control. No positive controls were available.

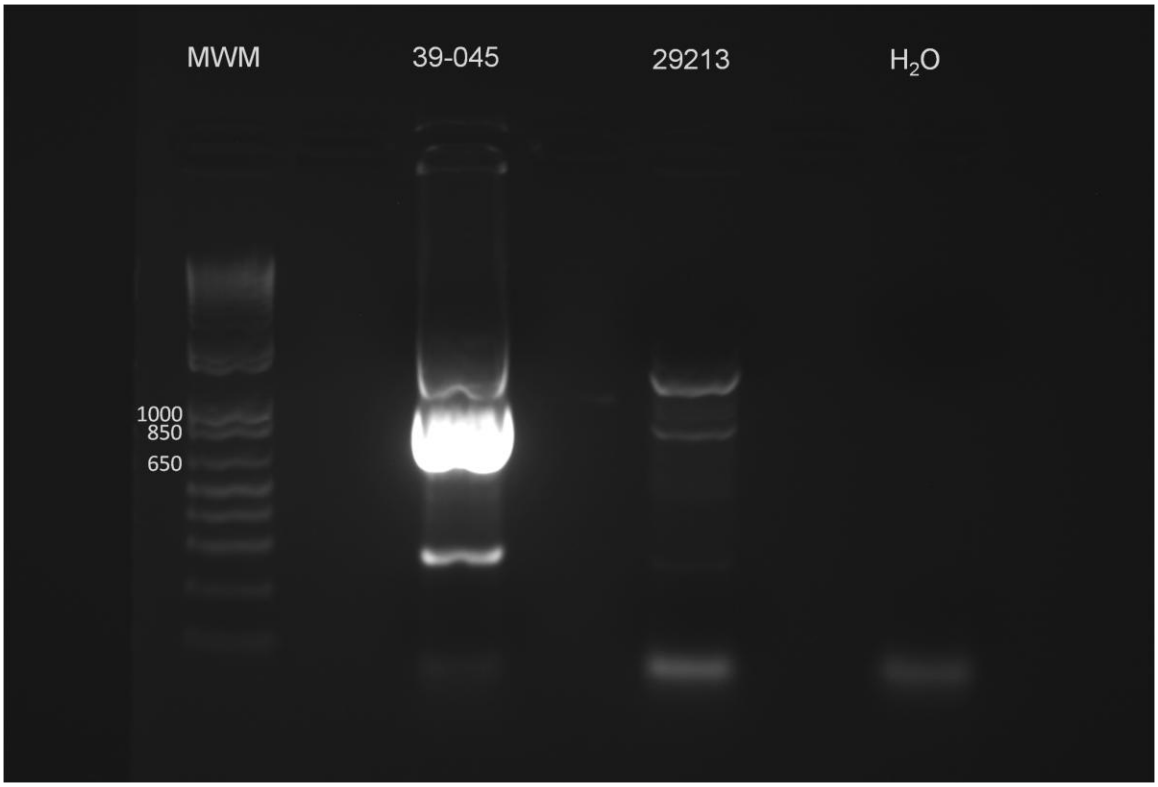

FIG. 4. Agarose gel electrophoresis patterns showing PCR amplification products for the native *ileS* gene using primers *ileS*-F1 and *ileS*-R1. MWM indicates 1 kb Plus molecular weight marker, 39-045 indicates methicillin-susceptible, mupirocin-resistant *S. pseudintermedius* isolate, 29213 indicates the methicillin-susceptible, mupirocin-susceptible *S. aureus* ATCC 29213used as a positive control, and H_2O indicates water with no template DNA, used as a negative control.

APPENDIX B

TABLES

TABLE 1. Classification of isolates by study

Study	Isolates	MRSP*	MSSP**	Dogs	Cats
Historic Clinical Data	202	75	127	159	6
Clinical Infections† (Sep. 2010-Feb. 2012)	195	58	137	162	3
Prevalence Study†† (Sep. 2010-Feb. 2012)	175	13	162	126	0
Totals	572	146	426	447	9

*MRSP = Methicillin-resistant *S. pseudintermedius*
**MSSP = Methicillin-susceptible *S. pseudintermedius*
†Isolates collected from patients with clinical infections concurrent with the period of the MRSP prevalence study conducted in patients presented for elective orthopedic procedures.
††Isolates collected from patients presented for elective orthopedic procedures.

TABLE 2. Number of *Staphylococcus pseudintermedius* clinical infection isolates by collection site

Collection Site	Totals	Subtotals
Skin	**95**	
Ear	**40**	
Wound	**53**	
Postoperative Infection	**42**	
Bone		12
Surgical Device		13
Surgical Incision		17
Urinary Tract	**88**	
Urine		66
Bladder Mucosa		12
Bladder Stone		7
Kidney		3
Respiratory tract	**16**	
Frontal Sinus		1
Larynx		3
Lung		1
Pharynx		1
ET Tube		2
Bronchial		2
Trachea		6
Reproductive Tract	**9**	
Penis		1
Prepuce		1
Vagina		4
Uterus		3
Other	**54**	
Abscess		8
Blood		3
Eye		3
Joint Fluid		4
Nasal		8
Liver		1
Other		20
Perianal		3
Peritoneal Cavity		3
Thoracic Cavity		1
Total	**397**	

TABLE 3. Primers used in this study

Target Gene	Primer	Sequence (From 5' to 3')	Reference
ileS2	mupA	TATATTATGCGATGGAAGGTTGG	(2)
	mupB	AATAAAATCAGCTGGAAAGTGTTG	(2)
	M1	GTTTATCTTCTGATGCTGAG	(25)
	M2	CCCCAGTTACACCGATATAA	(25)
IS257-*ileS2* Junctions	*ileS2*-5'	CCATGTCAACCCAGTATCC	(35)
	$IS257_F$	GGCATGGCGAAAATCCGTAG	(35)
	ileS2-3'	TCGGTGTAACTGGGGAATTA	(35)
	$IS257_R$	TGGCGTATTGATGAGACGTACATC	(35)
Internal Fragment of *ileS2* Upstream Junction	*ileS* 581F	GGAGAGAGCAATAATGAATCGGC	This Study
	ileS 1186R	CTTGAAAGGTCTTTGCTATATTCGT	This Study
ileS	*ileS*-F1	CGTGACCGTGGCGAATGGGT	(30)
	ileS-R1	GTATGCGGAATGATTGGCG	(30)